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**13. ABSTRACT** *(Maximum 200 Words)*

Our working hypothesis is that induced p120 loss in the breast will impair E-cadherin function leading to (1) severe adverse consequences to lobular-alveolar development, and (2) positive effects on tumorigenesis or tumor progression leading to increased invasion and metastasis. To determine the role of p120 inactivation in breast cancer, we have proposed to study the consequences of targeted p120 loss of function in the mammary glands of normal and transgenic mouse models for tumorigenesis and metastasis. The first aim proposes to use gene targeting to incorporate loxP sites at strategic locations in the p120 gene such that Cre recombinase-induced deletion of the intervening sequence will inactivate p120. We report here that the floxed p120 mouse has now been successfully generated. There are three male and two female mice, all of which contain the homologously recombined construct. This was the most difficult hurdle in the project. We can now proceed to target the p120 KO to the mammary gland and study in detail the outcome with respect to normal and cancerous behavior. The mouse will be invaluable for studying further the role of p120 in metastasis and breast cancer.

**14. SUBJECT TERMS**
Breast Cancer, p120-catenin, metastasis, E-cadherin, transgenics

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## Table of Contents

## INTRODUCTION

Accumulating evidence indicates that defects in the cytoplasmic mediators of cadherin function, the catenins, may account for cadherin dysfunction in carcinomas where E-cadherin expression is apparently normal. To determine the role of p120-catenin inactivation in breast cancer, we plan to study the consequences of targeted p120 loss of function in the mammary glands of normal and transgenic mouse models for tumorigenesis and metastasis. We have used gene targeting to incorporate loxP sites at strategic locations in the p120 gene such that Cre recombinase-induced deletion of the intervening sequence will inactivate p120. Mice containing this conditional allele ($p120^{flox}$) will be generated and crossed with WAP-Cre mice to target the conditional deletion to the breast. We will determine the effects of selective p120 loss in the breast with respect to lobular-alveolar development, cadherin function, tumor progression, and metastasis. Our working hypothesis is that induced p120 loss in the breast will impair E-cadherin function leading to (1) severe adverse consequences to lobular-alveolar development, and (2) an acceleration of tumorigenesis or tumor progression leading to increased invasion and metastasis. Understanding the role of p120 in these processes may lead to new strategies for pharmacologic intervention as a means of inhibiting metatasis.

## BODY

The statement of work for this grant is included below for reference. Figures associated with this work are shown in appendix A. There is now significant evidence that p120 loss occurs frequently in cancer and may contribute to tumor progression (Thoreson and Reynolds, 2002). Moreover, our recent data shows that p120 loss by mutation results in a significant reduction in the levels of E-cadherin and failure to organize normal epithelial structure (Ireton et. al., 2002). Thus, it is likely that the second part of our working hypothesis (see above) is likely to be born out by the experiments we are pursuing using the conditional p120 KO mouse generated during this funding period.

The most difficult hurdle for this proposal has been to generate the conditional p120 KO mouse. We reported previously on the successful completion of the targeting construct (Fig. 1A) and its successful introduction into ES cells by homologous recombination. Figures 1B and 1C show by southern blotting and PCR, respectively, that the floxed allele has recombined correctly into several ES cell lines. We also showed that addition of Cre *in vitro* results in correct recombination of the floxed p120 construct and loss of the sequence between the lox sites (Fig. 2). Our only modification of the original scheme was the insertion of frt motifs on either side of the neo cassette. In some instances, unscheduled splicing into the neo construct has been problematic for others. The modification made it possible to remove neo from the recombined p120 gene before moving the ES cells into mice, but increased the chances that too much manipulation of the ES cells would lead to damage that might prevent germline transmission. We screened 397 ES clones that potentially contained a homologously recombined allele. We then completed a two part screening process that initially identified the homologous recombinants, and subsequently isolated recombinants that had removed the frt cassette (Fig. 3). These accomplishments completed the work outlined below in Tasks 1 and 2.

In this last year of funding, we have accomplished the most difficult step of obtaining germline transmission of the floxed allele. There were several delays associated with problems in the mouse colonies at Vanderbilt. In addition, our first generation of chimeric mice were sterile (for reasons that are still not clear). However, a second generation of chimeric males have yielded entire litters of agouti pups, indicating that germline transmission has been achieved. We have analyzed the DNA from the agouti litter shown in to determine (1) whether the floxed alleles were successfully transmitted, and (2) whether the entire construct was present, including the lox sites flanking the introduced p120 sequence. Importantly, the PCR reactions demonstrate that five of the ten pups contain both these features (Fig. 4), indicating that we have succeeded in the completion of aim 1 of this grant. Of the five positive pups, three are male and two are female. Thus, Task 3 is now complete and we are breeding the mice to obtain the numbers we need to continue on with Tasks 4 and 5.

We had several problems, mostly due to an MSV infection in the mouse colony, and poor breeding conditions over the summer. These have now been successfully resolved, as evidenced by the birth of mice with the germline transmitted p120 allele. Overall, the project is in good shape and there are no major problems to report aside from the fact the project has taken longer than we anticipated. In retrospect, it has moved along

well and the delays we have experienced are relatively normal issues associated with this kind of work. We are pleased to report the final derivation of these mice, as this was the major goal of this proposal.

The reviewer has suggested that we obtain a no cost extension to finish this work. However, we fully intend to finish the work outlined in Tasks 4 and 5 and an administrative extension is not necessary. With the mouse in hand, we have the key reagent that will allow us to obtain NIH support in the form of an RO1, and I believe this is the best course of action for future progress. RO1 support will be necessary in order to support the significant breeding costs associated with the completion of these experiments. This was an essential part of the long term plan, as the Idea Award mechanism was not going to be enough by itself to support the costs of the mouse work. Recent developments in the p120/E-cadherin field indicate that this mouse will be invaluable for understanding the role of p120 in breast cancer metastasis, and we remain optimistic that these experiments will result in important progress in understanding the mechanisms underlying how and why breast metastases occur.

**STATEMENT OF WORK**
**Specific Aims 1 and 2: Role of the catenin p120 in Breast Cancer**

**Task 1:** Months 1 - 6. Generation of the knockin contruct designed to introduce Cre-lox sites into the introns flanking the sequence to be deleted.

A. Insert the 4.639 kb genomic p120 sequence containing exons 3 - 5 into pBS246 and sequence critical regions to verify absence of mutations.
B. Subclone the targeting arms (regions flanking exons 3 - 5) into sites flanking the Not I site in pBS to generate pBS-A/C.
C. Insert the pBS246 Not I cassette into pBS-A/C to generate the final targeting vector.

**Task 2:** Months 3 - 9. Generation of floxed p120 ES cell lines

A. Transfect ES cells with the Cre-loxP targeting construct, select transfectants in G418, and screen for homologous recombination by southern analysis.
B. Generate the appropriate recombination event by transient transfection of Cre recombinase, gancyclovir selection, and analysis by PCR.
C. Test the cell lines in vitro to verify the ability to induce p120 loss in the presence of Cre

**Task 3:** Months 9 - 15. Generate floxed p120 mice

A. Blastocyst injections by the Vanderbilt Transgenic Core and generation of several founder lines with germline transmission containing the heterozygous floxed p120 allele.

**Task 4:** Months 15 - 24. Generate homozygous $p120^{flox}$ / WAP-Cre mice

A. $p120^{flox}$ / + and $tg^{WAP\text{-}Cre}$ / + crosses to generate experimental and control mice.
B. Characterization of the resulting mice by PCR and southern blotting.

**Task 5:** Months 24 - 36. Determine the effects of p120 loss in normal and abberant breast function.

A. Determine effects of p120 loss on lobular-alveolar development and function.
B. Test the long term effects of p120 loss in mammary tumor progression and malignancy by crossing the $p120^{flox}$/WAP-Cre mice with TAg and/or MMTV-MT mouse models for mammary carcinogenesis.

**KEY RESEARCH ACCOMPLISHMENTS** (bullet format)

*Cloned and sequenced the genomic p120 DNA
*Inserted lox-P sites into introns flanking exons 1 – 7.
*Introduced frt sites on either side of the neo cassette.
*Ligated fragments of the above constructs to generate the targeting vector, containing floxed p120 sequences and a frt flanked neo cassette.
*Successfully tested the removal of the genomic sequence between the loxP sites in vitro.
*Generated 397 ES clones containing the transfected targeting construct.
*Identified several clones containing homologously recombined p120 floxed allele.

*Generated chimeric mice from ES cells containing the introduced p120 floxed allele.
*Obtained germline transmission of the floxed p120 allele. We have thus generated a key mouse model for determining the consequences of p120 loss with respect to breast cancer.

## REPORTABLE OUTCOMES

The generation of mouse models (particularly condition KO mice) generally takes 2 to 3 years. Although the project fell behind the expected schedule, the successful test of the recombination reaction makes it unlikely that the construct will fail in the mouse. We now have germline transmission of the floxed allele, which essentially means we have successfully generated our p120 condition KO mouse model. Therefore, I remain extremely optimistic, even though we do not yet have a reportable outcome in the form of a completed manuscript. In retrospect, the time frame estimated for breeding of the mice was unrealistic. However, with the mouse in hand, we are in an excellent position to obtain NIH RO1 funding, which will allow us to complete these experiments. The student responsible for most of this work (Michael A. Davis) has attended two meetings, at which the work was reported in abstract form (see appendix).

## CONCLUSIONS

We have completed a significant body of work resulting in the successful generation of a mouse model where we can target p120 gene deletion to the mammary gland. Moreover, we have demonstrated successful removal of the floxed sequence by Cre. There are no major changes in the original plan, and we are now addressing the really interesting biological problems relevant to breast cancer metastasis. Our mouse model is likely to be extremely important for understanding metastasis in breast and other cancers. Recent progress in the pathology literature shows frequent loss of p120 in breast and other types of carcinomas (reviewed in Thoreson and Reynolds, 2002) and our recent data shows that p120 and E-cadherin are likely to act together as tumor and metastasis suppressors (Ireton et. al., 2002). By inducing p120 loss in the mammary gland, we can now directly test the hypothesis that p120 is functionally relevant as a tumor suppressor.

## REFERENCES:

Thoreson MA and Reynolds AB. 2002. Altered Expression of the Catenin p120 in Human Cancer: Implications for Tumor Progression. Differentiation. 70:583-589.
Ireton RC, Davis MA, van hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L, Gilbert G, van Roy F, and Reynolds AB. 2002. A novel role for p120 catenin in E-cadherin function. J Cell Biol.159:465-476.

## APPENDICES:

Appendix A: Figures referred to in the text.
Appendix B: Reportable outcomes.

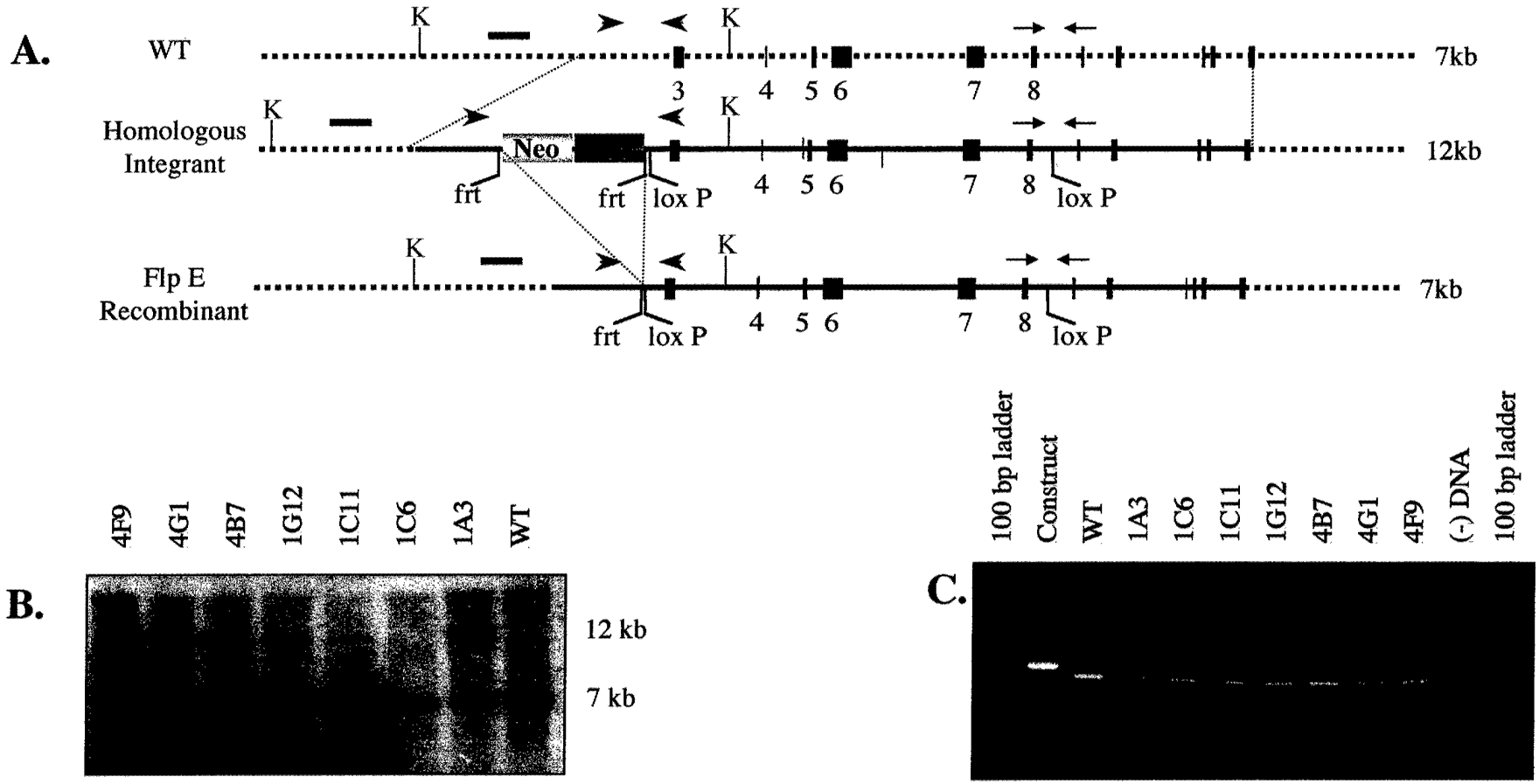
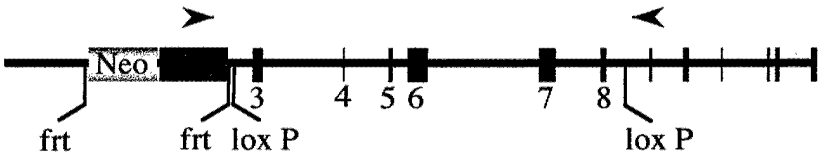



Fig. 1 Homologous integration of targeting construct. (A) Schematic representation of the wild-type (WT), homologously integrated, and Flp E-recombined alleles. Genomic DNA is represented as a dotted line; the position of the targeting construct is indicated with a solid line. Exons are shown as solid blue boxes. The Neomycin (Neo) and Thymidine Kinase (TK) selection cassettes are illustrated as yellow and pink boxes respectively. For Southern blot analysis, the probe used is indicated by Red bars, Kpn I (K) restriction sites are shown as blue vertical lines, and the sizes of the expected bands are listed to the right of the schematic. Arrows represent primers used in assaying for the 3' Lox P site, and arrowheads represent primers used in screening for Flp E recombination. (B) Southern blot analysis of wild type (WT) ES cells and of seven recombinant ES cell clones isolated after neomycin selection. (C) PCR analysis of the 3' Lox P site for the seven ES cell clones. The targeting construct and wild type DNA serve as a positive controls for the presence and absence of the 3' Lox P site respectively.

**Fig. 2 A.**




**B.**

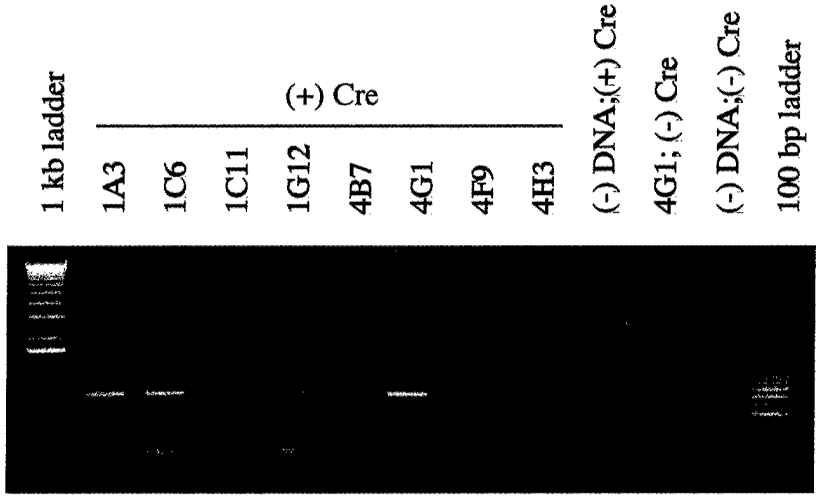



Fig. 2. The Lox P sites are functional in vitro. (A) Schematic indicating the position of the primers (arrowheads) used to test Lox P function. (B) PCR assay demonstrating functionality of Lox P sites. 5 μg DNA from ES cell clones was incubated with 1 μ g Cre recombinase in a 50 μl reaction for 30 minutes at 37°, then the enzyme was heat inactivated at 80 ° for 20 minutes. 10 μl of each sample was used to assess Cre-mediated recombination by PCR. Clone 4H3 does not contain the 3' Lox P site and therefore should not undergo Cre-mediated deletion. As 10 kb of DND lies between the Lox P sites in unrecombined DNA, no band is expected for unrecombined DNA due to the short duration of the PCR extension cycle.`

**Fig. 3.**

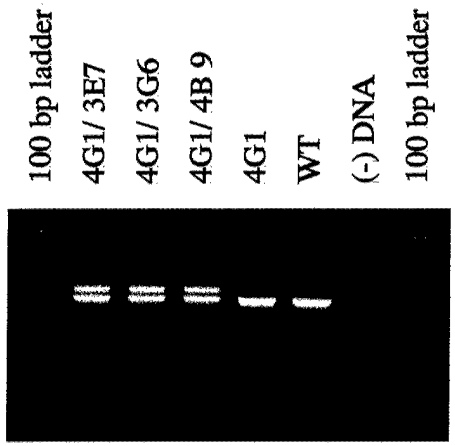



Fig. 3. Flp E-mediated recombination. ES cell clone 4G1 was electroporated with Flp E recombinase, and cells were selected using Gancyclovir. DNA from three recombinant ES cell clones, the parental ES cell clone 4G1, and from wild type ES cells was assayed by PCR for Flp E recombination. Due to the presence of a frt site and a Lox P site, alleles undergoing Flp E recombination will be 120 bp larger than the wild type allele. Thus Flp E recombined cells, which contain one wild type allele and one recombinant allele, will appear as a doublet. As the unrecombined allele, containing the 5kb Neo-TK cassette, is too large to generate a band with the short PCR extension time used here, no band is expected for this allele.

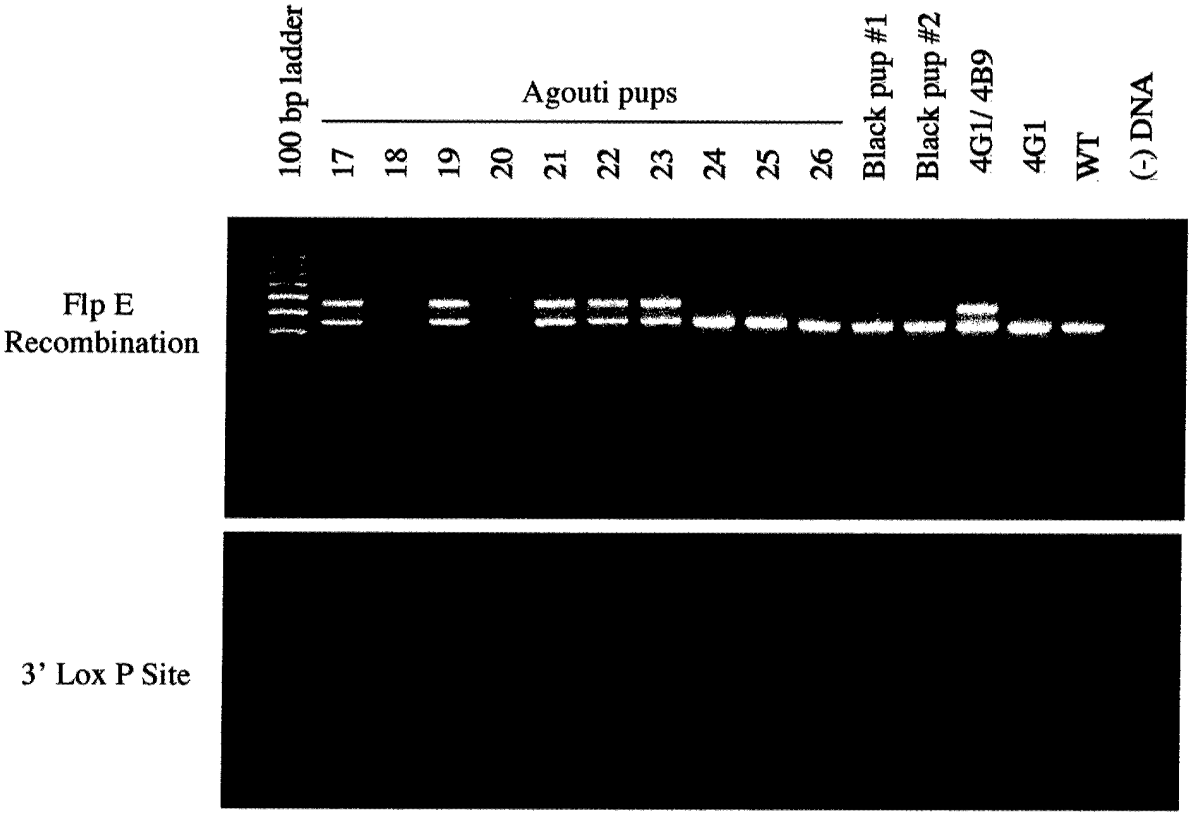



Fig. 4. Germline Transmission of targeted allele. DNA isolated from tail clippings of ten agouti pups was assayed for Flp E recombination and for the presence of the 3' Lox P site as before. DNA from the ES cell clone 4G1/4B9 was used as a positive control for Flp E recombination, whereas tail DNA from two black pups and DNA from wild type ES cells served as a negative control. The faint upper band seen with 4G1 DNA in the Flp E recombination assay is background, as these cells have not been treated with Flp E. The upper band seen for 4G1 DNA in the 3' Lox P site assay is expected.as these cells contain the targeting construct.

## Appendix B

Davis MA and Reynolds AB. Mammary-Specific Deletion of $p120^{ctn}$. Presented at Vanderbilt University Department of Cancer Biology Departmental Retreat. Lake Barkley State Resort Park, Cadiz, Kentucky. November 15, 2002.

Davis MA and Reynolds AB. Mammary-Specific Deletion of $p120^{ctn}$. Presented at The third Era of Hope meeting for the Department of Defense Breast Cancer Research Program. Orange County Convention Center, Orlando, Florida. September 25-28, 2002.

Davis MA and Reynolds AB. Targeted Deletion of p120. Presented at The American Association for Cancer Research, Edward A. Smuckler Memorial Workshop on Pathobiology of Cancer. Keystone Resort, Keystone, Colorado. 2001